**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

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| Application of: | Choong-Chin Liew | Examiner: | Jennifer Ann Dunston |
| Serial No.: | 10/812,702 | | |
| Filed: | March 30, 2004 | Group Art Unit: | 1634 |
| Titled: | METHOD FOR THE DETECTION OF CORONARY ARTERY DISEASE RELATED GENE TRANSCRIPTS IN BLOOD | Conf. No.: | 5754 |

**Commissioner for Patents**
**P.O. Box 1450**
**Alexandria, VA 22313-1450**

**DECLARATION OF Jun Ma UNDER 37 C.F.R. §1.132**

Sir:

I, **Jun Ma**, Ph.D., hereby declare that:

1. I received a Ph.D. degree from the Dept. of Clinical Medicine and Pathobiology at the University of Toronto in 2003, and B.Sc. degree from the Dept. of Biology at the University of Science and Technology of China in 1997.

I have approximately ten years of working experience in functional genomics, and I am particularly experienced in the field of gene expression profiling of blood for purposes of disease biomarker identification.

I have been working at GeneNews as a research scientist in functional genomics since October 13, 2003 and have specifically been involved in the area of identifying biomarkers for coronary artery disease.

I have personally reviewed all the data and experiments described in this, my declaration.

List of Publications:

**Ma J**, Dempsey AA, Stamatiou D, Marshall KW, Liew CC. Identifying leukocyte gene expression patterns associated with plasma lipid levels in human subjects.Atherosclerosis. 2007;191(1):63-72.

Liew CC, **Ma J**, Tang HC, Zheng R, Dempsey AA. The peripheral blood transcriptome dynamically reflects system wide biology: a potential diagnostic tool. J Lab Clin Med. 2006;147(3):126-132.
**Ma J**, Liew CC. Gene profiling identifies secreted protein transcripts from peripheral blood cells in coronary artery disease. J Mol Cell Cardiol. 2003;35(8):993-998.
Claudio JO, Liew CC, **Ma J**, Heng HH, Stewart AK, Hawley RG. Cloning and expression analysis of a novel WD repeat gene, WDR3, mapping to 1p12-p13. Genomics. 1999;59(1):85-89.

2. I have read the Office Action mailed April 16, 2007 in the above-referenced patent application. I understand that the Examiner has raised concerns, which are addressed in the instant declaration, with respect to the reproducibility and validation of the results disclosed in the instant specification, and the utility of the gene CRTAM as an indicator of coronary artery disease.

3. As a scientist skilled in the area of gene expression profiling, I submit that levels of CRTAM-encoded RNA have been experimentally shown to be significantly higher in blood of CAD patients relative to healthy control subjects so as to demonstrate utility as a test useful for providing an indication of coronary artery disease as noted in **currently amended** claims 49.

**Levels of CRTAM-encoded RNA are significantly higher in blood of coronary artery disease patients versus healthy control subjects – validation of CRTAM as CAD biomarker in blood via quantitative RT-PCR**

Attached as Exhibit "A" to this Declaration is a disclosure and analysis of post-filing experiments performed by Applicant. I have reviewed and analyzed the data which has been generated regarding levels of CRTAM-encoded RNA and have found that levels of CRTAM-encoded RNA in blood samples from patients with coronary artery disease (CAD) were significantly higher relative to healthy control subjects, as determined via quantitative RT-PCR (QRT-PCR).

I have confirmed that the quantitative RT-PCR experiments were performed as described under Materials and Methods in Exhibit "A", below, in order to reproduce and expand upon results obtained using microarray hybridization analysis described in the specification (Example 21) disclosing differential expression of CRTAM-encoded RNA between blood samples from CAD patients and healthy control subjects. As shown in Tables 1 and 2 and Figure 1 of Exhibit "A", the average level of CRTAM-encoded RNA in samples from 19 CAD patients, as determined via QRT-PCR, was found to be 2.9-fold higher than that of 14 healthy control subjects tested, with the difference in expression levels being statistically significant ($p<0.05$). The signal intensity data for each sample was analyzed via the ROC curve approach to determine the optimal expression level threshold to differentiate between levels of CRTAM-encoded RNA in samples from CAD patients versus healthy control subjects. As can be seen in Table 2 of Exhibit "A", 16 out of 19 CAD samples scored above the classification threshold value, while 9 out of 14 healthy control subject samples scored below the classification threshold value. These results hence signify that CRTAM-encoded RNA levels are significantly higher in blood of CAD patients relative to healthy subjects, and that analysis

of such levels resulted in an indication of CAD in a human test subject with a specificity of 64% and a sensitivity of 84%.

In view of the above, I submit that the results disclosed in the specification have been reproduced using even greater numbers of samples, and that the CRTAM gene continues to demonstrate utility as a test useful for providing an indication of coronary artery disease.

5. I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that wilful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardise the validity of the application or any patent issuing thereon.

Jun Ma, Ph.D. 

Date Aug 28, 2007

# EXHIBIT "A"

**TABLE 1.** Quantitative RT-PCR analysis of CRTAM-encoded RNA levels in blood of CAD patients vs healthy control subjects. The relative fold-change threshold used for sample classification was: 1.3. Data were obtained as described under Materials and Methods, below. Median fluorescence of controls normalized to a value of "1".

| Sample origin | Sample ID | Level of CRTAM-encoded RNA (fold-change fluorescence relative to median of controls) | Sample classification (fold-change ≤ 1.3: "non-CAD"; fold-change > 1.3: "CAD") |
|---|---|---|---|
| **CAD patients** | CAD1 | 0.283 | "non-CAD" |
| | CAD2 | 0.288 | "non-CAD" |
| | CAD3 | 0.893 | "non-CAD" |
| | CAD4 | 1.325 | "CAD" |
| | CAD5 | 1.416 | "CAD" |
| | CAD6 | 1.430 | "CAD" |
| | CAD7 | 1.632 | "CAD" |
| | CAD8 | 1.731 | "CAD" |
| | CAD9 | 1.941 | "CAD" |
| | CAD10 | 2.080 | "CAD" |
| | CAD11 | 2.080 | "CAD" |
| | CAD12 | 2.191 | "CAD" |
| | CAD13 | 2.206 | "CAD" |
| | CAD14 | 2.744 | "CAD" |
| | CAD15 | 3.390 | "CAD" |
| | CAD16 | 3.438 | "CAD" |
| | CAD17 | 6.283 | "CAD" |
| | CAD18 | 8.947 | "CAD" |
| | CAD19 | 10.714 | "CAD" |
| **Healthy control subjects** | CTRL1 | 0.203 | "non-CAD" |
| | CTRL2 | 0.408 | "non-CAD" |
| | CTRL3 | 0.638 | "non-CAD" |
| | CTRL4 | 0.672 | "non-CAD" |
| | CTRL5 | 0.767 | "non-CAD" |
| | CTRL6 | 0.822 | "non-CAD" |
| | CTRL7 | 0.924 | "non-CAD" |
| | CTRL8 | 0.950 | "non-CAD" |
| | CTRL9 | 1.267 | "non-CAD" |
| | CTRL10 | 1.522 | "CAD" |
| | CTRL11 | 1.874 | "CAD" |
| | CTRL12 | 2.292 | "CAD" |
| | CTRL13 | 2.439 | "CAD" |
| | CTRL14 | 2.517 | "CAD" |

**TABLE 2.** Analysis of QRT-PCR data of Table 1, above, for CAD detection. Statistical analysis was performed as described under Materials and Methods, below.

| | |
|---|---|
| **Fraction CAD samples above threshold fluorescence** | 16/19 |
| **Fraction control samples below threshold fluorescence** | 9/14 |
| **Accuracy** | 25 / 33 x 100 % = 76 % |
| **Specificity** | 9 / 14 x 100 % = 64 % |
| **Sensitivity** | 16 / 19 x 100 % = 84 % |
| **Average fold-change (CAD/control)** | 2.9 |
| ***p*-value** | < 0.05 |

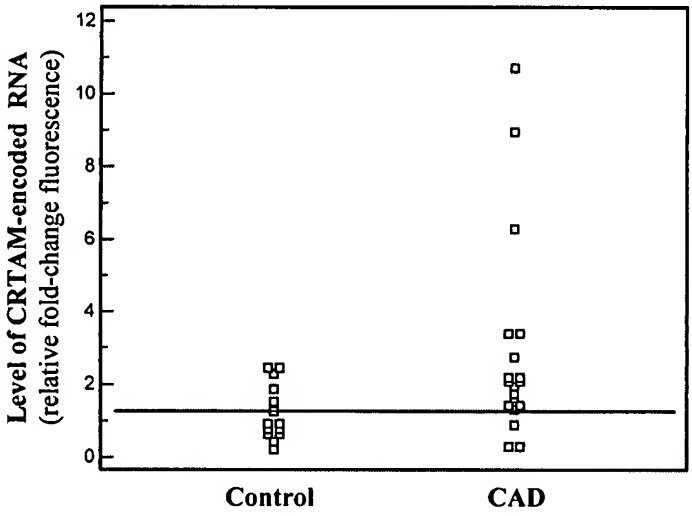


**FIG. 1.** Levels of CRTAM-encoded RNA in blood samples from CAD patients are on average 2.9-fold higher ($p<0.05$) than those of healthy control individuals, as determined via real-time quantitative RT-PCR. The horizontal line represents the classification threshold value of 1.3, determined via ROC curve analysis. The data points are plotted relative to median control levels, normalized to a value of "1". Data were obtained as described under Materials and Methods, below, and correspond to data shown in Table 1 above.

## Materials and Methods:

*Blood RNA Isolation*: Samples were obtained from 22 patients diagnosed with single or multi-vessel CAD prior to angioplasty, and from 14 healthy control subjects. All participants provided written informed consent. Approximately 10ml of blood was collected from each participant, using a Vacutainer™ tube (Becton Dickinson, Franklin Lakes, NJ). Samples were treated with lysis buffer (1.6 mM EDTA, 10 mM KHCO3, 153 mM NH4Cl, pH 7.4), followed by centrifugation. The total RNA in the resulting pellet was extracted with Trizol® Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instruction. The quality of RNA samples was assessed on an Agilent Bioanalyzer 2100 using RNA 6000 Nano Chips (Agilent

Technologies, Palo Alto, CA), and the quantities of RNA were measured by UV spectrophotometry (Beckman-Coulter DU640).

*Real-time QRT-PCR:* Real-time QRT-PCR was used to measure levels of CRTAM-encoded RNA in blood samples from CAD patients and healthy control subjects. First strand cDNA was synthesized from 1μg total RNA using the ABI High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) in a volume of 100μl, consisting of 10μl 10x RT buffer, 4μl 100mM dNTP mix, 10μl 10x RT random primers and 5μl Multi-scribe reverse transcriptase (50U/μl). Real-time PCR was performed on an ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). PCR was performed in a reaction volume of 25μl consisting of 12.5μl 2X SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 5μl of 5pmol primer mix (forward primer, AGCCCACCAGTACTGTCTCAG; reverse primer, ACTGAGGATTTGCTTCGGTGGT; both primers are optimized for an annealing temperature of 56°C), and 2.5ng first strand cDNA. The PCR cycling protocol used is as follows: (1) 50°C, 2 min; (2) 95°C, 10 min; (3) 40 cycles of 95°C, 15 sec; 60°C, 1 min; and (4) determining the dissociation curve from 60°C to 95°C. The beta-actin gene or 18S rRNA was used as the housekeeping gene for normalization.

*Determination of classification thresholds and evaluation of sensitivity and specificity:* Receiver operating characteristic (ROC) curve analysis [Pepe MS. The Statistical Evaluation of Medical Tests for Classification and Prediction. Oxford: Oxford University Press; 2003; Metz CE. Basic principles of ROC analysis. Semin Nucl Med 1978; 8: 283-98; Swets JA. Measuring the accuracy of diagnostic systems. Science 1988; 240: 1285-93; Zweig MH, Campbell G. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. Clin Chem 1993; 39: 561-77] was used to analyze the signal intensity data for each gene to determine the optimal expression level classification threshold for classifying levels of CRTAM-encoded RNA in the samples as being characteristic either of CAD patients or of healthy control subjects. Receiver operating characteristic curve analysis was used to evaluate the TPF (true positive fraction; sensitivity) and FPF (false positive fraction; 1-specificity). Receiver operating characteristic curves were generated using MEDCALC software.

*Statistical analysis:* A Mann-Whitney rank sum test was used, using the fold-change values, to test for the statistical significance of the difference in RNA levels between the disease and healthy control groups. Fold change was calculated using the following formula: $2^{-\Delta\Delta Ct}$,

where ΔΔCt was calculated by subtracting the mean ΔCt value of the control samples from the ΔCt of each sample for each gene. Statistical analysis was performed using SigmaStat v3.0 (SPSS Scientific, Chicago, IL).